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14. ABSTRACT

The purpose of this study is to define correlations between indices of brain dysfunction, such as functional MRI (fMRI) and the neuropsychological testing abnormalities, with oxidative phosphorylation (OXPHOS) defects in children with autism spectrum disorders (ASD). This study is an essential step in identifying such a phenotypic subtype, being able to perform large-scale epidemiological studies using more widely available measures, and ultimately being able to implement clinical trials for new pharmaceutical agents emerging for treatment of the OXPHOS defects which could significantly improve the functioning of children with ASD with this defect. Data acquisition is ongoing and larger numbers of subjects are needed before meaningful conclusions can be drawn from the study.

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Autism, functional MRI, mitochondria, mitochondrial disease

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Introduction

Although the precise frequency of mitochondrial defects in autism are not known, it is hypothesized that significant numbers of individuals with autism and autistic spectrum disorders (ASD) harbor oxidative phosphorylation (OXPHOS) defects important to ASD disease pathogenesis and/or functioning. These OXPHOS defects are identifiable in muscle, fibroblasts and EBV transformed lymphocytes. Pathogenic mutations in OXPHOS genes are predicted to be observed in patients with ASD at higher rates than in the general population. We propose that these OXPHOS defects correlate with indices of brain dysfunction such as functional MRI (fMRI) and neuropsychological testing abnormalities and define a specific subtype of children with ASD. The proposed study is an essential step in identifying such a phenotypic subtype, being able to perform large-scale epidemiological studies using more widely available measures, and ultimately being able to implement clinical trials for new pharmaceutical agents emerging for treatment of the OXPHOS defects which could significantly improve the functioning of children with ASD with this defect.

Body

Brief background:

Mitochondria are cytoplasmic structures with an inner and outer membrane separated by an intermembrane space. Oxidative phosphorylation (OXPHOS) is critical to cellular function as the primary source for energy (ATP) in most cell types, the control point for cellular redox, and as a control point for essential metabolic and signaling pathways that range from the synthesis of pyrimidines for the regulation of apoptosis. Substrates for ATP generation are derived primarily from glycolysis and fatty acid oxidation.

OXPHOS uses about 95% of the oxygen delivered to tissues, producing most of the ATP required by cells. Expression of genes involved in the OXPHOS pathway and the assembly of the five OXPHOS enzyme complexes Complex I (CI), Complex II (CII), Complex III (CIII, CIV and CV) within the inner mitochondrial membrane is a highly ordered and coordinated process directed by 37 genes in the mitochondrial DNA (mtDNA) and as many as 1,500 genes in the nuclear DNA (nDNA)[1,2].

Over 50 pediatric and adult diseases are caused by mutations in a heterogeneous array of OXPHOS genes coded by either the nDNA or the mtDNA. Genetic defects producing mitochondrial dysfunction include: (1) inherited mutations in nDNA or mtDNA genes. (2) Sporadic mutations occurring during embryogenesis that are systemic or confined to specific tissues such as skeletal muscle. (3) Somatic mutations occurring through life due to aging, free radical damage, and exposure to environmental toxins or certain medications. Defects in OXPHOS have a broad array of cellular consequences including abnormal cellular calcium ($Ca2+$) regulation, impaired ATP generation, enhanced apoptosis, and increased free radical production. [3- 6] In fibroblast cell lines harboring pathogenic mutations in CI genes, CI dysfunction causes depolarization of the mitochondrial membrane potential, resulting in a decreased supply of mitochondrial ATP to the Ca2+-ATPases that control intracellular Ca2+ stores. Ca2+ content

of these stores is then reduced, particularly in the endoplasmic reticulum. [7] Defects in any of these functions can lead to disease.

Most energy used for neuronal activity is expended as a result of the postsynaptic neuronal depolarization and to a lesser extent the action potentials generated. [8] OXPHOS uses approximately 95% of the oxygen delivered to tissues, thus making fMRI an important tool for non-invasive investigation of mitochondrial dysfunction. The energy cost arises from information transfer and its integration postsynaptically. Substrate delivery for energy metabolism is increased along with increased local blood flow in conjunction with to neurotransmitter action (local signaling). Reduced oxygen extraction as occurs with mitochondrial dysfunction leads to an increase in the ratio of oxy- to deoxyhemoglobin during neuronal activation.

Mitochondrial disease produces detectable fMRI abnormalities. For example, in Friedreich ataxia, a mitochondrial disease caused by abnormal iron incorporation into OXPHOS enzyme active centers [9], fMRI studies during motor tasks show cortical hypoactivity in a pattern consistent with the mitochondrial dysfunction.[10] fMRI abnormalities are well described in ASD and correlate with clinical features. Cortical hypoactivity in ASD includes fusiform gyrus which is associated with poor facial recognition [11] and anterior cingulate cortex which is related to inflexible and repetitive behavior [12]. Although the neuroanatomical substrates of the ASD phenotype are characterized, studies correlating fMRI findings with biochemical or molecular defects are lacking.

Task 1: (Specific Aim I, human subjects, Months 1-30) Assessment of Neuropsychological Functioning. In order to make appropriate statistical analyses among neuropsychological data, fMRI data and laboratory data, the data analysis will extend to the end of the study (month 36).

During the current year (Y3) we have continued to clinically evaluate subjects and enroll new ones. During Year 3 work we enrolled nine additional subjects (five just in 4th quarter). Six subjects completed all aspects of the study, but one of these six could not complete the fMRI scan or testing due to their level of functioning. Another five subjects are still in process and will be returning for additional evaluation.

Over the study to date, we have now completed 26 (three additional during 4th Quarter) of the 33 Autism Diagnostic Interview – Revised (ADI-R) for the total sample, with three being currently scheduled (See Figure 1 for study overview). In addition, we completed the in-person diagnostic play session (ADOS) on 19 subjects (four during 4th Quarter).

As previously documented, the pattern of results from different ASD measures on children with histories of the ASD diagnosis is presented in the total sample flow chart (Figure 1 below), and the subjects meeting different combinations of ADI-R or ADOS (or both) criteria are provided in Table 1. To date, only five children with mitochondrial disease have met <u>both</u> the full ADI-R and ADOS criteria as proposed from the 25 subjects who have had the ADI-R, and the 19 who have also had the ADOS evaluation. Fourteen of the nineteen subjects who have completed both the ADI-R and ADOS procedures meet one or the other diagnostic criteria.

Now that a no-cost extension (NCE) has been granted, we will complete the process for reimbursement of per diem for some of these families who have to travel across the country to participate in this study.

Table 1: Summary of ADI-R and ADOS progress in the 33 subjects that have enrolled in the study.

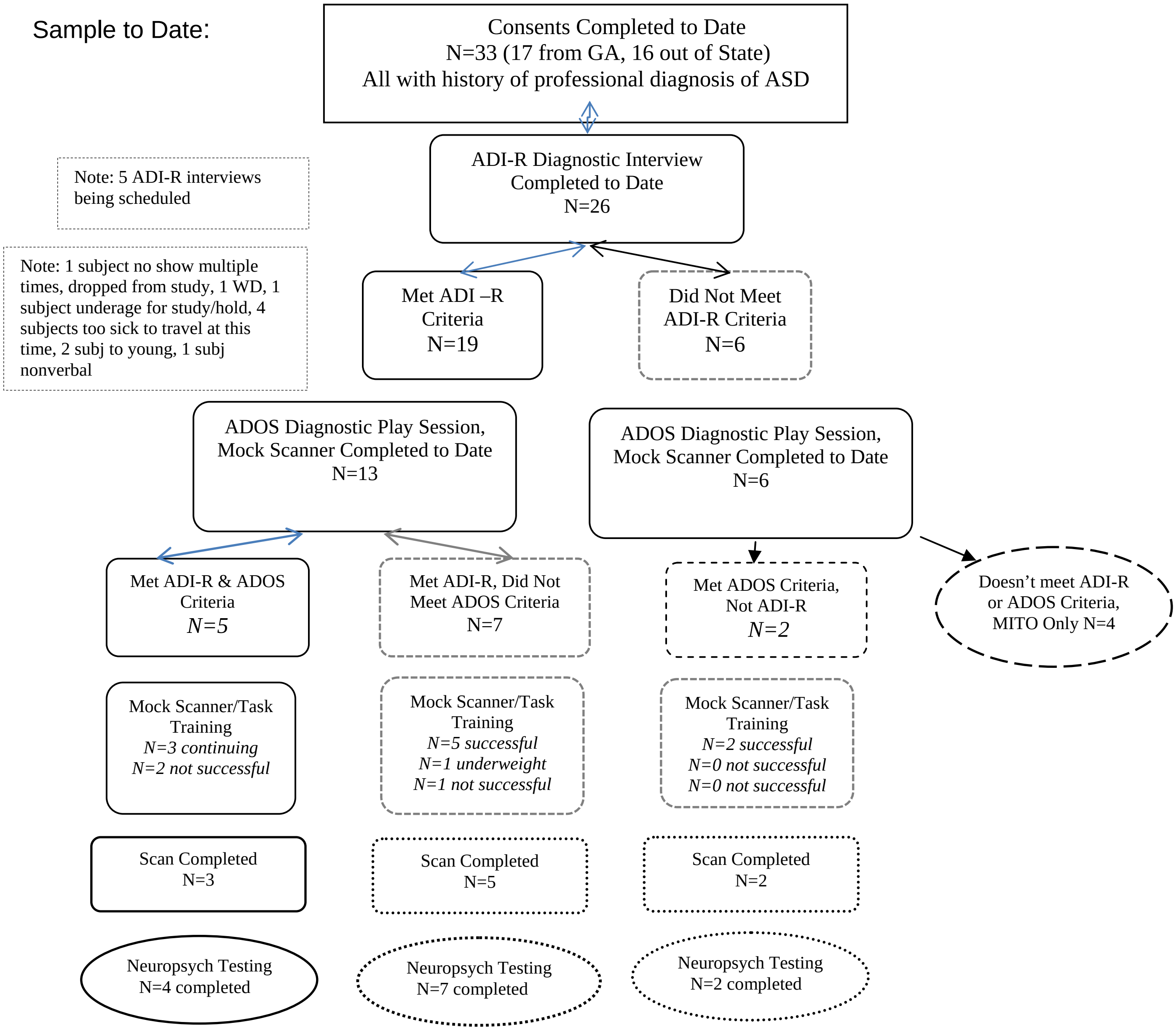
Includes subjects who met either ADI or ADOS criteria	consent obtained	ADI completed	ADOS completed	meets full study criteria	Meets ADI-R criteria	Meets ADOS criteria	Doesn't meet either ADI-R or ADOS criteria
Year 1 as of 6/30/2011	10	4	0	0	0	0	0
Year 2 as of 6/30/2012	14	14	9	2	13	4	2
Year 3 as of 6/30/2013	**9**	**8**	**10**	**3**	**6**	**3**	**2**
Totals	33	26	19	5	19	7	4

Table 2: Comparing Subjects who meet criteria for ADI-R & ADOS (N=5) vs. those who only meet criteria for ADI-R (not ADOS) (N=7) and example results from Neuropsychological and Adaptive Behavior Measures for subjects who have completed any testing to date.

Subject Characteristics (avg (std, range))	Sample Meets ADI-R & ADOS Criteria	Sample Meets ADI-R Criteria Only
Age (in years)	12.0 (2.6)	8.7(3.5)
ADI-R Social	20.2 (7.8)	20.3(7.7)
ADI-R Communication	17.0 (4.9)	17.2(8.5)
ADI-R Repetitive Stereotyped Behavior	7.2(4.1)	6.3(2.7)
ADI-R Abnormal/Dev Problems Evident at or before 36 months	2.4(2.1)	3.5(0.9)
ADOS Communication	4.6 (2.5)	0.8(0.8)*
ADOS Reciprocal Interaction	10.2 (3.6)	1.2(0.4)*
ADOS Stereotyped Behavior	2.4 (2.7)	0.0 (0.0)
WIAT Word Reading	90.2(20.1)	121.0(31.3)
DAS Verbal SS	93.3 (41.1)	116.8(27.8)
DAS Working Memory SS	72.3(10.1)	102.0(19.6)*
DAS Processing Speed SS	70.7(23.6)	94.8(11.6)*
NEPSY Faces Delayed Recall SS	7.7(6.4)	8.5(2.5)
NEPSY Inhibition-I Combined SS	5.0(2.6)	6.5(1.3)
NEPSY Fingertip Tapping Combo SS	2.3(2.3)	8.0(3.6)*
Vineland Communication	74.0 (12.2)	95.8(20.8)
Vineland ADL	63.0(3.5)	82.2(11.2)*
Vineland Socialization	59.7(9.9)	72.8(13.6)*
Vineland Adaptive Behavior	64.3(7.6)	80.8(11.4)*
Vineland Maladaptive Behav Score(raw)	18.7(8.3)	14.5(9.7)
		*p<.05 t-test between groups

Figure 1: Flow chart summarizing testing for the 33 subjects that have signed consents for the study.

Sample to Date:



Task 2: (Specific Aim II, human subjects, Months 1-30) Neuroimaging: In order to make appropriate statistical analyses among neuropsychological data, fMRI data and laboratory data, the data analysis will extend to the end of the study (month 36).

Data acquisition and analysis will continue throughout the study. To date, we have had five children who met both ADI-R and ADOS diagnostic criteria, three had completed all components of the study (one this quarter), including the fMRI scan. Two subjects who met both criteria have completed all aspects except the scan, and we will continue to work with one of them in the mock scanner to see if they will be able to be successfully scanned in the future. We do not expect the other to be able to scan.

As mentioned previously, because there are a number of cases that continue to have a documented history of receiving autism or ASD diagnoses at younger ages but do not meet both criteria for current autism/ASD diagnosis, we have begun to look at the symptoms that seem to be differentiating the children with mito disease and ASD from those with pure mito disease. *This is a different comparison then we had originally planned for this project.* Our original plan was to compare ASD+Mito with children with just ASD. Given that all of our subjects to date have Mito disease, but there is a range of ASD symptoms/behaviors depending on the measures and timing of the assessments, we propose that we reorient our study focus and analysis to compare children with ASD+Mito to those with just Mito. This is also because the number of children who have been given biopsy for mitochondrial disease who now meet *ASD only criteria* (no Mito) have not been identified as was originally expected (also related to the issues of early ASD history in their records but actual currently meeting the ADI-R and ADOS diagnostic criteria). Again, because of the diagnostic and developmental issues we have a number of interesting contrasts to make depending on the measures we use to classify a child as ASD. Given our current sample, if we used the most 1) ***strict criteria*** for ASD (current and historical, **both** ADI-R and ADOS positive), compared to any child with mito regardless of their independent ADI-R or ADOS results, we would have five ASD+Mito subjects meeting strict criteria, and 17 'Mito' subjects for comparison. More realistic would be to use a 2) ***mixed criteria*** for ASD (ADI-R positive **or** ADOS positive), which would yield 14 ASD+Mito subjects compared to four pure 'Mito' (no positive ADI-R or ADOS). We could also look at each measure only as the ASD criteria, 3) ***ADI-R positive criteria*** would yield 19 ASD+Mito subjects compared to six 'Mito' subjects; and a 4) ***ADOS positive criteria*** would result in seven ASD+Mito subject compared to 11 'Mito' subjects.

Now that the NCE is approved, we will complete the process to modify our original subject inclusion criteria from requiring both ADI-R and ADOS criteria, to allowing either (ADI-R or ADOS criteria) to be included in the remainder of the study and to be scanned. In addition, we are requesting to modify our design so that we now will compare children with ASD and mito disease to those with no ASD who have mito disease only. We currently have four children who do not meet any of the ASD criteria at this time, but who have biopsy diagnosed mito disease and we believe we can expand this sample. We believe this modification of sample comparisons will add to our knowledge about these complex set of disorders. Given the spectrum of ASD criteria results from our current sample, we believe that this change provides significant benefit for addressing our original aim of determining the similarities/differences between children with and without ASD and mito disease. We are requesting that these changes be approved so that we may modify our IRB to recruit a wider range of subjects that meet this goal.

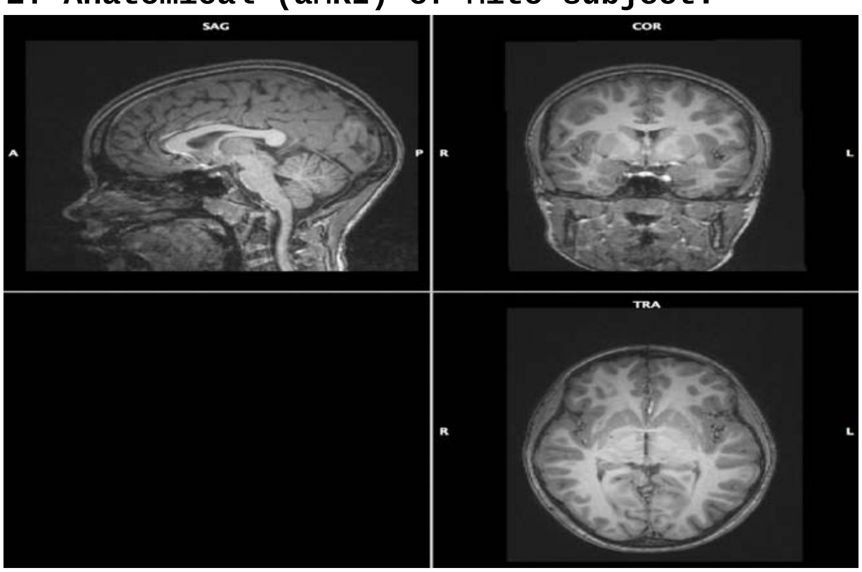
Table 2 presents some beginning analysis comparing those children who meet both ADI-R and ADOS criteria to those who only meet ADI-R criteria. As can be seen (acknowledging the small sample sizes and t-test analysis involved) these two groups do not differ on the mean ADI-R measures presented, suggesting that their parents rate them the same. But our independent researcher's behavioral observations and ratings of these children on the ADOS show significant differences across Communication and Reciprocal Interaction domains, but no significant difference in

Stereotyped Behaviors (although clearly the Both group has higher levels). In general, the Both group has lower neuropsychological functioning across almost all measures, although they all do not reach statistical significance. In general, the Both group appears to be more severely impaired compared to the ADI-R criteria only group. Overall, such very preliminary results may exemplify the spectrum and dimensional aspects of the ASD disorder, and the revised sampling and analysis approach we are suggesting would be able to better address such 'levels of severity' factors actually better then what was originally proposed. This is particularly interesting given our previously described study comparing our ADI-R results with those from another study of children matched for age with ASD but no mito. According to caregiver report on the ADI-R, it appears that children diagnosed with comorbid ASD and mitochondrial disease presented, on average, with less severe social deficits during their early social development; future research will examine specific social impairments that are different among children with comorbid ASD and mitochondrial disease. All of these results suggest that these children with ASD (again depending on how defined) and Mito may represent the spectrum between typically developing children and more classic ASD children who appear to have more severe difficulties.

Table 3: Subjects who have completed components of protocol, includes subjects who meet either ADI-R or ADOS criteria.

Includes subjects who met either ADI or ADOS criteria	parent measures completed	mock training completed	Nback training completed	NP testing completed	scan completed	study participation completed
Totals	12	12	12	13	10	13

Figure 2: Anatomical (aMRI) of Mito subject.



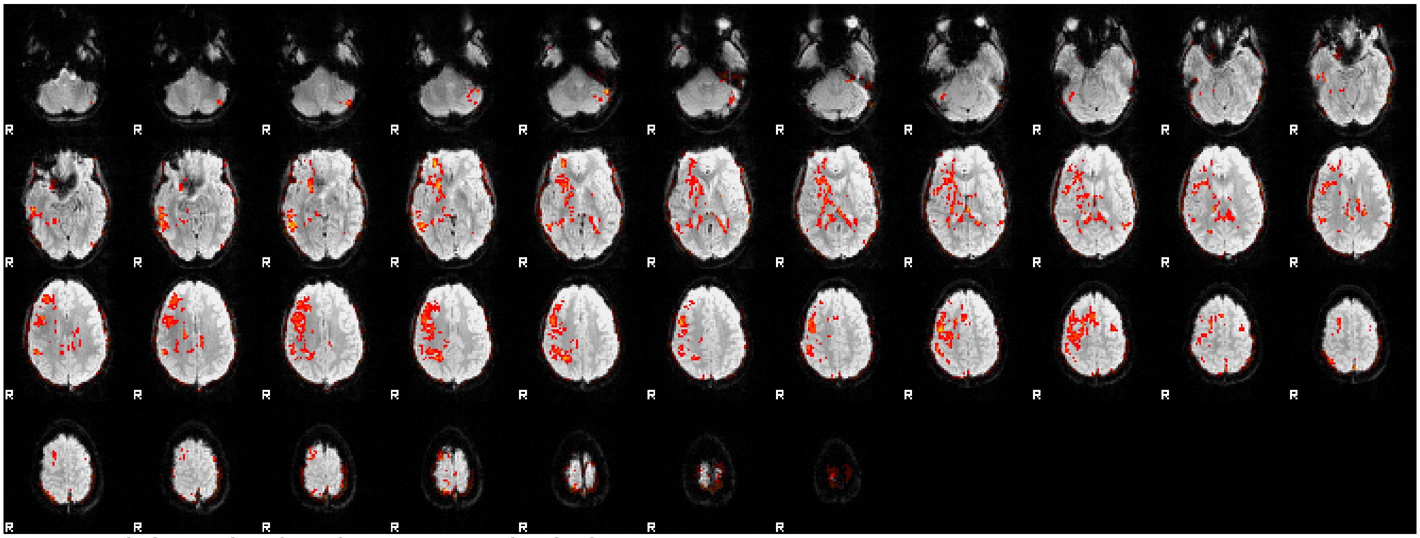
Data analysis of the ten completed fMRI scans has begun during the quarter. Movement analysis shows that seven of the scans are very good without much movement, three have some mild movement which we are working

on some correction to see if most of it is useable. We are in the processes of evaluating the activation fMRI results from these scans at this time, preliminary approach and results are shown below.

fMRI Analysis methods

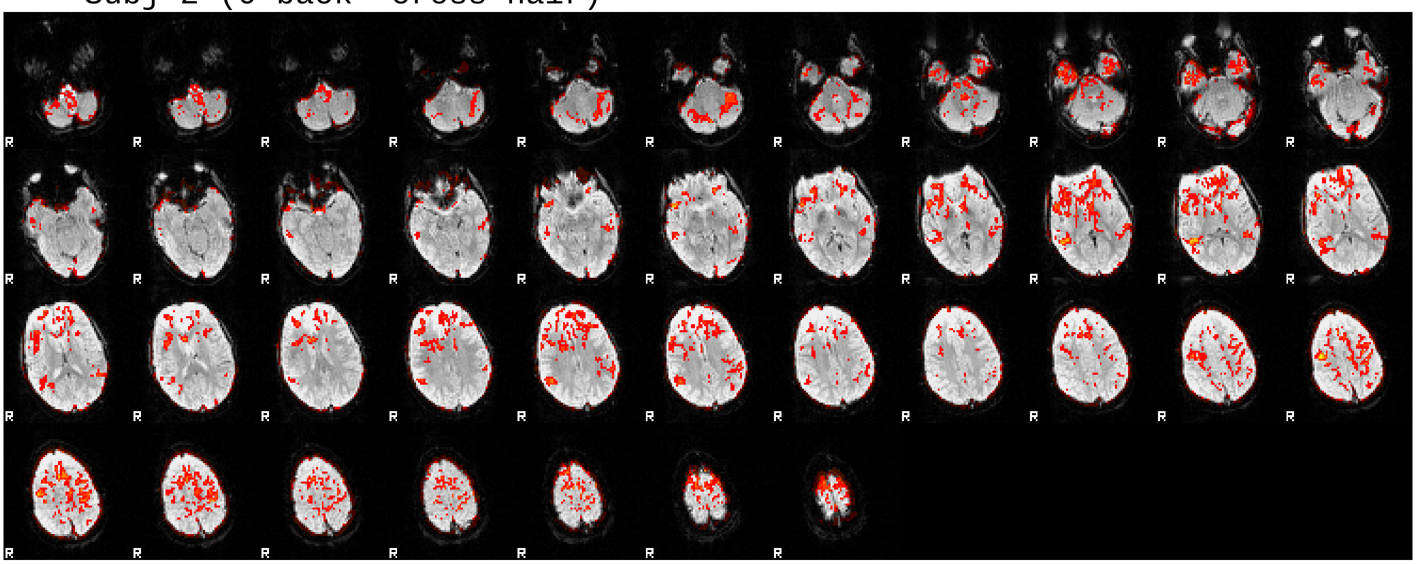
FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 6.00, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Z (Gaussianised T/F) statistic images were thresholded using clusters determined by **Z>1.65** and a (corrected) cluster significance threshold of P=0.05(13).

<u>0 back-Cross Hair</u>

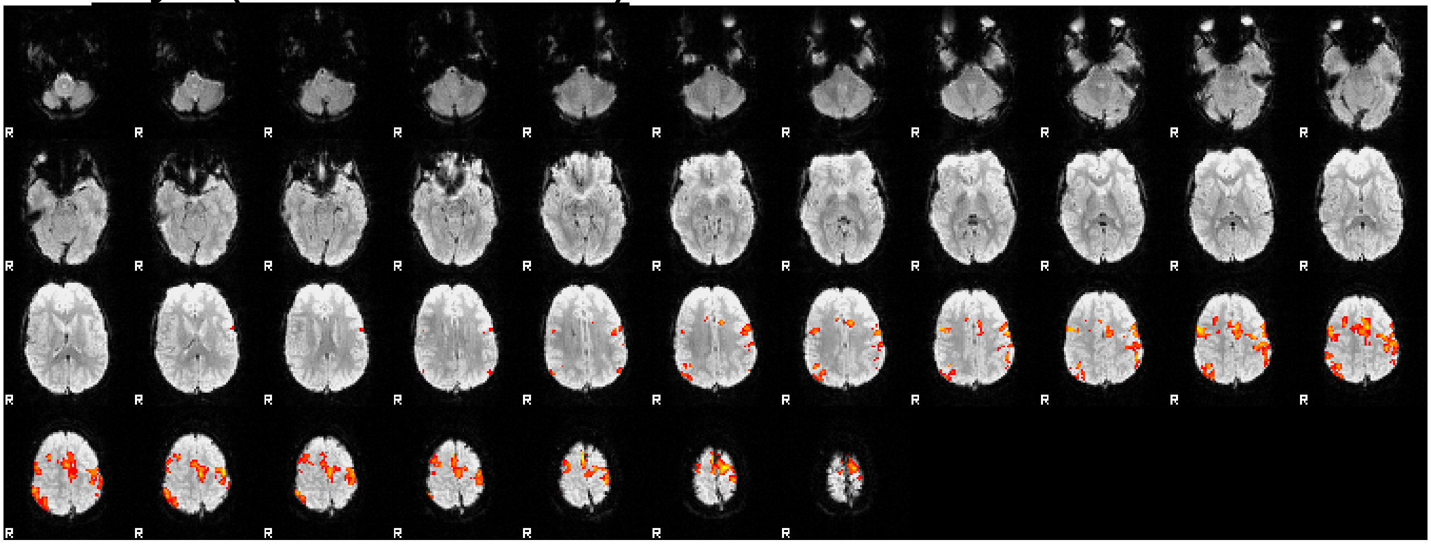


Subj 1 (0 back- cross hair)

Subj 2 (0 back- cross hair)



Subj 3 (1 back – cross hair)



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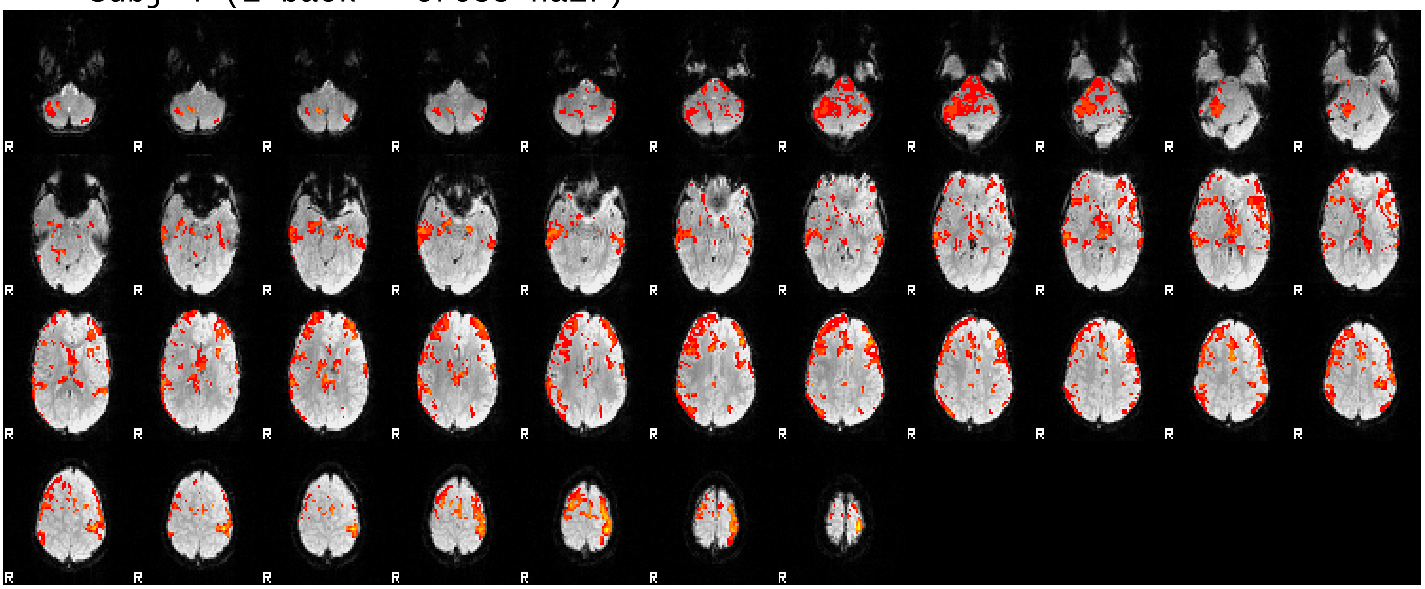
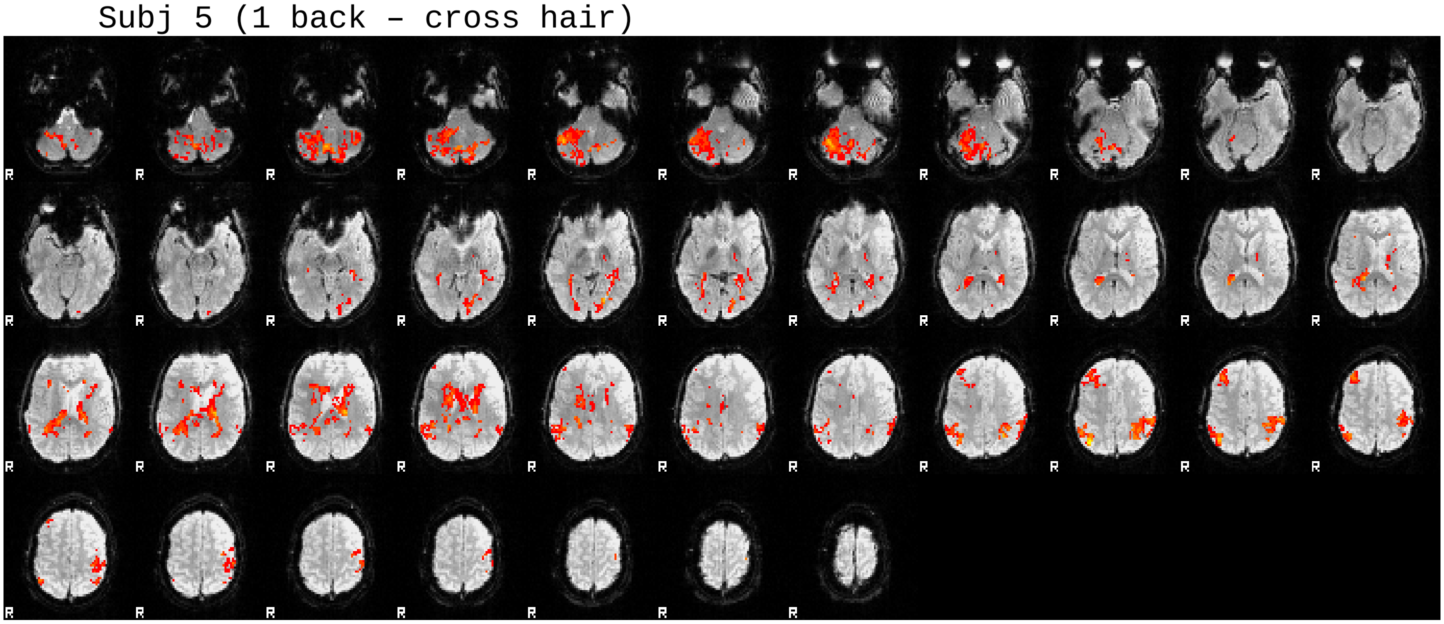


Subj 5 (1 back – cross hair)



Task 3: (Specific Aim III, Banked samples that include muscle, fibroblasts, and EBV transformed lymphocytes) Months 1-30. Although data analysis will be proceeding during the study, the last six months are reserved for assessing the data obtained from all specific aims (tasks).

As detailed in our recent quarterly report, we continue to make progress on this specific task. These types of analyses are complex, requiring comparison of patients with autism to various categories of normal control cell lines and disease controls. A variety of classes of mutations are being studied in order to understand how the various mitochondrial disease mechanisms affect the results of each test. Sample testing is currently underway and data analysis will be performed when sufficient numbers are obtained. We expect a majority of this analysis to occur during the last six months of this study.

Task 4: (Specific Aim 4, Banked DNA Analysis) Months 12-30

This task relies heavily on Task 3 in order to determine which genes to analyze for each patient. While sample testing is underway, data analysis will not be performed until sufficient numbers are obtained in order to draw meaningful conclusions. We expect a majority of this analysis to occur during the last six months of this study.

Key Research Accomplishments:

1) Data has provided a new, modified subject recruitment and classification approach which provides a better data driven approach to the complexities of diagnosis ASD in children with mito disease.
2) Nine new subjects submitted their consent, eight had their ADI-R completed, and eight had their ADOS completed during the year.
3) The completed fMRI scans have begun systematic activation analysis, as have other aspects of the data being collected.
4) Analysis of the N-back task completed during the fMRI scans has begun.
5) Manuscripts are currently being written that will reference this grant. One paper focuses on the relationship between neuropsychological test results and serum lactate/pyruvate ratios that documents strong correlations. Another paper uses the early history of these children and their current ADI-R & ADOS results to try to put together a better understanding of how these children are similar/different from ASD children that do not have OXPHOS disease. A third paper will be written on the subjects that do not qualify for this study. It will focus on laboratory and fMRI data in subjects that have OXPHOS disease and do NOT show signs of ASD. This study will provide an interesting and meaningful contrast to the data collected in this study.

Reportable Outcomes

None at this juncture in the study.

Conclusions

We continue to make progress in several aspects of this study during the past year, and believe the new no-cost extension (NCE) will allow us to increase our samples of children with ASD and Mito. We are requesting a change in our original subject classification/ASD criteria and sampling to address the complexities found in defining ASD in this sample, and comparing the ASD+Mito subject to Mito only subjects.

A continuing challenge for the study is the availability of subjects who have to travel a great distance and the extra expenses they incur if they are going to participate in the study. With the new NCE we will finalize our protocol to allow families who have expressed interest in the study but who cannot afford the extra days travel expenses to obtain some per diem reimbursement if they participate during their next visit to Atlanta. We have developed a separate consent form for this purpose, and this will be available so that parents can plan on visiting in the coming months. The study protocol continues to be a challenge for these children and their families and most subjects who have completed it have needed at least two days (with breaks, etc.) to be able to be successful at minimum.

In summary, the study protocol is a very challenging protocol for these children and their families, but we are progressing and gathering unique data from those that have participated. We are addressing the significant diagnostic challenges by asking for a modification of the original inclusion criteria so that children who meet either of the widely used diagnostic measures (ADI-R, ADOS) can participate in the study. This will provide a more generalizable sample of children with a history of autism/ASD and current symptomatology based on different criteria.

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Appendices

None.